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Divergent responses of biological nitrogen fixation in soil, litter and moss to temperature and moisture in a karst forest, southwest China

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ABSTRACT

Free-living biological N_2 fixation (BNF) in soil, litter and moss is an important pathway for external nitrogen (N) inputs to unmanaged terrestrial ecosystems. However, how free-living BNF responses to temperature, moisture and their interaction have not been well understood, especially in the subtropical regions. Here we investigated the responses of BNF in soil, litter and moss to temperature (15, 25 and 35 °C) and moisture (low, intermediate and high levels) over a one-week period under laboratory conditions. The samples were collected in a subtropical karst forest, southwest China. We found that the responses of BNF in moss, litter and soil to temperature and moisture were divergent. Litter and soil BNF rates increased with temperature under the three moisture levels. However, moss BNF rates were decreased, not changed or increased by warming under low, intermediate and high moisture levels, respectively. At each temperature, the BNF rates increased with moisture for moss and litter. However, the patterns of moisture responses were different at the three temperatures for soil BNF, which was not changed and increased by moisture at 15 °C and 25 °C, respectively; and was greatest under intermediate moisture, but lowest under low moisture at 35 °C. The temperature sensitivity of BNF was generally highest for litter, intermediate for soil and lowest for moss. In contrast, the moisture sensitivity of BNF was generally highest for moss, intermediate for soil, and lowest for litter. Our findings suggest that different temperature or moisture sensitivities of BNF for the three substrates should be integrated into terrestrial ecosystem models in order to better predict N inputs via BNF under climate change.

1. Introduction

Nitrogen (N) is the major limiting element for net primary productivity (NPP) in terrestrial ecosystems (LeBauer and Treseder, 2008). The dynamics of N availability is usually expected to have substantial impacts on an ecosystem's structure, functions and processes (Xia and Wan, 2008; Liu and Greaver, 2009, 2010; Bobbink et al., 2010; Niu et al., 2016). In addition, N availability is crucial in determining long-term terrestrial carbon (C) sequestration under climate change (Hungate et al., 2003; Luo et al., 2004). Therefore, understanding the dynamics of ecosystem N inputs, which relate directly to N availability, is undoubtedly important for predicting climate change-C cycle feedbacks.

Biological N_2 fixation (BNF hereafter) is a major pathway of external N inputs to unmanaged terrestrial ecosystems (Fowler et al., 2013; Houlton and Morford, 2015). BNF may be performed by symbiotic and free-living organisms (Reed et al., 2011). According to Reed et al. (2011), symbiotic BNF is defined as BNF occurring via relationships between plants and the N_2-fixing symbionts in plant root nodules either as *Rhizobia* or *Frankia*. Free-living BNF, includes BNF carried out by cyanobacteria in association with moss or by other free-living diazotrophs in soil and litter (Reed et al., 2011). The free-living BNF rates vary from 0.01 to 60 kg N ha^{-1} yr^{-1} depending on climate and ecosystem type, but are generally lower than symbiotic BNF rates (Cleveland et al., 1999; Reed et al., 2011). However, for those ecosystems lacking (or with a low abundance of) N_2-fixing plants, free-living BNF may be the dominant pathway of external N inputs.

Free-living BNF is affected by a few abiotic factors (Reed et al., 2011), including the climate related factors, e.g., temperature (Bjerke et al., 2003; Sorensen and Michelsen, 2011; Rousk et al., 2017), and precipitation (Gundale et al., 2012b; Whiteley and Gonzalez, 2016; Rousk et al., 2017). Global warming and subsequent changes in

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precipitation patterns have been well documented and will continue in the near future with some regions becoming wetter while others are predicted to become dryer (IPCC, 2014). How BNF responds to climate change could directly affect plant production and ecosystem C sequestration (Hungate et al., 2003; Smith et al., 2014). However, the current treatment of BNF in terrestrial models is entirely empirical, and mostly based on the empirical relationships between BNF rates and NPP, evapotranspiration (ET) or other variables (William et al., 2015). There is large uncertainty in terms of how BNF will respond to climate change, which unavoidably increases the uncertainty in predicting climate change-C cycle feedbacks (Hungate et al., 2003; Thornton et al., 2007).

So far, most of the studies investigating free-living BNF responses to temperature and/or moisture have focused on BNF of bryophyte-cyanobacteria associations, especially moss BNF (Bjerke et al., 2003; Gundale et al., 2012b; Lindo et al., 2013; Rousk et al., 2017). According to these studies, BNF of bryophyte-cyanobacteria associations may be enhanced (Gundale et al., 2012a; Lett and Michelsen, 2014; Rousk et al., 2017), remain unchanged (Bjerke et al., 2003; Deslippe et al., 2005; Sorensen and Michelsen, 2011; Whiteley and Gonzalez, 2016) or decreased (Sorensen et al., 2012) by warming. In contrast, moisture has often been found to have a strong impact on BNF with BNF rates increasing with moisture (Gundale et al., 2009; Whiteley and Gonzalez, 2016; Rousk et al., 2017). To our knowledge, only one study has investigated the response of soil BNF to warming under controlled conditions, and found that soil BNF increased exponentially with increasing temperature (Diáková et al., 2016). However, the available studies, which investigated the responses of free-living BNF to climate change, have generally been conducted in boreal or arctic regions. In these regions, the mean annual air temperature is much lower than 10 °C. However, some of the studies show that the optimum temperature for free-living BNF is between 20 °C and 30 °C (Gundale et al., 2012a; Rousk et al., 2017), but others have found that the optimum temperature differs according to cyanobacteria-moss associations. For example, Gentili et al. (2005) found that *Nostoc* sp. had a maximum BNF rate at 13 °C while *Calothrix* sp. had maximum at 30 °C. It remains unclear whether free-living BNF in tropical or subtropical regions responds to climatic changes in a similar manner. Furthermore, since no study has simultaneously explored the responses of BNF in soil, litter and moss to climate change, it remains unclear whether BNF in these ecosystem compartments respond similarly or not.

In order to address the above uncertainties, the responses of soil, litter and moss BNF to temperature and moisture were investigated under laboratory conditions. Samples were collected from a secondary forest with a very limited abundance of N_2-fixing plants in a subtropical karst region, southwest China. This region has experienced an increase in temperature over the past decades and is predicted to become warmer in the 21st century (IPCC, 2014). Data from 70 meteorological stations show that different areas have higher risk of extreme drought or wet events in the southwest China (Liu et al., 2014). Nevertheless, how climate change influences BNF has not been investigated in this region. Therefore, the main objectives of the present study were to address: (i) How does BNF in moss, litter and soil respond to variation in temperature and moisture? (ii) Are there interaction effects of temperature and moisture on BNF? (iii) Are there differences in the temperature sensitivity or moisture sensitivity of BNF?

2. Materials and methods

2.1. Sampling site

Samples were collected from a secondary forest at Mulun National Nature Reserve (107°53′-108°05′ E, 25°06′-25°12′ N) in Guangxi Zhuang Autonomous Region, southwest China. The region is located in the subtropical humid forest life zone and has a monsoon climate. Mean annual air temperature is 17.8–21.1 °C, with the lowest monthly mean in January (7.8–11.2 °C) and the highest in July (25.8–29.0 °C). Mean annual precipitation ranges from 1346 to 1498 mm with a distinct seasonal pattern. The period from April to August is the wet season (accounting for about 71% of the annual precipitation) while September to March is the dry season (accounting for about 29% of the annual precipitation). Total atmospheric N deposition is about 37 kg N ha^{-1} yr^{-1} (Zhu et al., 2015). The studied area is characterized by a typical karst landscape with gentle valleys flanked by steep hills. The bedrock is mostly limestone nested with dolomite. The soil is classified as a calcareous lithosol (limestone soil) according to the FAO/UNESCO classification system (Li et al., 2017). Soil depth varies from 0 to 80 cm in the valley and ranges from 0 to 30 cm on the slopes. The selected forest site was about 35 years old at an elevation of 420 m. The forest had naturally regenerated from an abandoned maize-soybean rotation field. The dominant tree species included *Cladrastis platycarpa* (Maxim.) Makino, *Liquidambar formosana* Hance, *Cryptocarya chinensis* (Hance) Hemsl., *Eurycorymbus cavaleriei* (Levl.) Rehd. et Hand. and *Itoa orientalis* Hemsl. Moss species occur on rocks, dead trunks, downed wood and the forest floor (Fig. S1). The soil texture was silt loam.

2.2. Sample collection and experimental design

Samples of soil, litter and moss (hereafter referred to as substrate) were collected from six randomly selected plots (20 m × 20 m each) within an area of 100 m × 100 m in July 2016. Ten locations of 50 cm × 50 cm were randomly selected in each plot. At each location, all the bulk leaf litter was collected. Soil samples from the surface 2 cm were collected with a soil corer (5 cm in diameter) upon removal of the litter layer. The green branches of the dominant moss (*Hypnum plumaeforme*) were collected from the rock surface with a knife. Upon transporting to the laboratory, the soil (or litter/moss) within a plot was mixed thoroughly to form a composite sample, and left in the laboratory for one or two days to lower the moisture to the low level of respective substrate (Table 1) if necessary. Some physicochemical properties of soil, leaf litter and moss are presented in Table 2.

The experiment employed a full factorial design, including three air temperature levels (15, 25 and 35 °C) and three levels of moisture for each category of substrates (Table 1). The three air temperature levels were within the range of air temperature reported in this region with mean minimum daily air temperature of 8 °C (January) – 25 °C (July) and mean maximum daily air temperature of 15 °C (January) – 33 °C (July) across the year. The low, mid and high levels of moisture for soil and litter were adjusted to be close to the lowest (28.4% for soil, and 36.4% for litter; moisture is based on dry weight basis hereafter), average (35.1% for soil, and 59.7% for litter) and maximum (44.2% for soil, and 80.2% for litter) gravimetric moisture values recorded in four field campaigns in January, April, July and October. For moss, the low

Table 1
Temperature (°C) and moisture (%) treatments applied to moss, litter and soil, respectively, in the incubation experiment.

	Moss		Litter		Soil	
	Temperature	Moisture	Temperature	Moisture	Temperature	Moisture
Low level	15	74.3	15	19.7	15	28.8
Mid-level	25	84.5	25	53.9	25	37.2
High level	35	93.6	35	73.6	35	42.8

Table 2
Physicochemical properties of soil, litter and moss (mean ± standard error, n = 3).

	Bulk density g cm^{-3}	pH	Water content %	Organic C g C kg^{-1}	Total N g N kg^{-1}	Total P g P kg^{-1}
Soil	1.1 (0.1)	6.4 (0.1)	37.4 (2.1)	50.3 (0.3)	5.2 (0.1)	1.6 (0.1)
Litter	NA	NA	20.0 (1.9)	412.9 (3.5)	18.7 (0.3)	1.6 (0.1)
Moss	NA	NA	62.9 (2.8)	349.4 (8.4)	28.3 (0.2)	3.3 (0.1)

Note: NA denote not available. Water content of the substrates is reported as gravimetric water content.

level of moisture was close to the average value recorded in the four field campaigns (71.0%), while the high level of moisture was similar to the highest value recorded in the four field campaigns (93.0%). After moisture adjustment, the moss, litter and soil samples were allocated to each of the nine treatments (Table 1). Each treatment included 6 replicates. The amount of sample for each replicate was 10 g for soil, 2 g for litter and 1 g for moss. For each replicate, the sample was placed in a 250 ml conical flask for incubation. The jars were covered with parafilm® M (Laboratory Film, USA) to prevent evaporation of water but allow air exchange.

The samples were incubated under three levels of air temperature achieved using three climate chambers (GZH 450, Zhonglang Tech, China), respectively. The photosynthetically active photon flux density was kept at 100 μmol m^{-2} s^{-1} for the three chambers. Deionized water was sprayed if water loss was detected by weight.

2.3. Measurements of nitrogen fixation rates

Nitrogen fixation rates were measured one week following treatment using the acetylene reduction method (Hardy et al., 1968). The flasks were sealed with rubber stoppers. Then 10% of the headspace of each flask was replaced with acetylene (cylinder acetylene with 99.99% purity) using a gas-tight syringe. The acetylene was purified by passing through 98% sulfuric acid and 5 M sodium hydroxide solution before use (Keuter et al., 2014). After acetylene injection, the flasks were put back into the corresponding climate chamber and incubated for another 24 h. Then a 20 ml gas sample was collected from the headspace of each flask and transferred into a 12 ml pre-evacuated glass vial (Labco Exetainer, Labco Limited, UK). In addition, ethylene (C_2H_4) concentrations in acetylene only flasks and sample only flasks were estimated to ensure that the ethylene measured in the sample flasks was derived from nitrogenase enzyme activity. Ethylene in the gas samples was analyzed using a gas chromatograph (Agilent GC 7890A, Agilent, USA) equipped with a flame ionization detector (FID).

Following gas analysis, litter and moss samples were dried at 65 °C for 48 h, and soil samples were dried at 105 °C for 24 h and weighed. Ethylene production rates or acetylene reduction activity (ARA, nmol C_2H_4 g^{-1} dry mass d^{-1}) were calculated from the slope of the regression line between ethylene concentrations and time. The temperature sensitivity (Q_{10}) of BNF, the factor by which the BNF rate increases for a 10 °C increase in temperature, was calculated according to equation (1) (Li et al., 2013):

$$Q_{10} = \left(\frac{ARA_{T_2}}{ARA_{T_1}}\right)^{10/(T_2-T_1)} \quad (1)$$

where T_2 and T_1 are the incubation temperatures (°C). Similarly, the moisture sensitivity (RM_{10}) of BNF, the factor by which the BNF rate increases for a 10% increase in moisture, was calculated according to equation (2):

$$RM_{10} = \left(\frac{ARA_{M_2}}{ARA_{M_1}}\right)^{10/(M_2-M_1)} \quad (2)$$

where M_2 and M_1 are the incubation moisture (%) levels.

2.4. Data analyses

Data were tested before analyses for assumptions of normality and homogeneity of variances. Three-way analysis of variance (ANOVA) with Tukey's *post-hoc* test was used to examine the effects of substrate type (moss, litter and soil), temperature (15 °C, 25 °C and 35 °C)/or temperature range (ΔT, i.e., DT1: from 15 to 25 °C, and DT2: from 25 to 35 °C), moisture (LM, MM and HM)/or moisture range (ΔM, i.e., DM1: from LM to MM, and DM2: from MM to HM), and their possible interactions on BNF rate, Q_{10} or RM_{10}. Two–way ANOVA was used to examine the effects of temperature (or temperature range), moisture (or moisture range) and their possible interactions on BNF rate, Q_{10} or RM_{10} for each substrate type. One-way ANOVA with Tukey's *post-hoc* test was performed to examine the effect of temperature (or moisture) on BNF rate under each moisture (or temperature) level on BNF rate for each substrate, or the effect of moisture (or temperature) on Q_{10} (or RM_{10}) for each temperature (or moisture) range per substrate. Independent-Samples T test was used to examine the effect of temperature (or moisture) range on Q_{10} (or RM_{10}) under each moisture (or temperature) level per substrate. The effects were considered to be significant if $P < 0.05$. The statistical analyses were performed using SPSS 16 (SPSS Inc., Chicago, IL, USA).

3. Results

The three-way ANOVA showed that there were significant effects of substrate type, temperature, moisture and their interactions on BNF rate ($P < 0.001$) (Fig. 1a). Overall, the BNF rate was highest in moss, intermediate in litter and lowest in soil (Fig. 1a). The averaged BNF rate in moss was 2.0 and 21.4 times that in litter and soil, respectively. As the temperature or moisture increased, the overall BNF rate increased (Fig. 1a). Substrate type, ΔT and moisture individually or interactively had significant ($P < 0.05$) or marginally significantly ($P < 0.1$) effects on Q_{10} except the interactive effect of substrate type × ΔT ($P > 0.1$) (Fig. 1b). The overall Q_{10} value of BNF rate was highest for litter, intermediate for soil, and lowest for moss, and was higher in DT2 than in DT1, or was higher under MM than that under LM (Fig. 1b). There were significant effects of substrate type, temperature, ΔM and their interactions on RM_{10} ($P < 0.01$) (Fig. 1c). The value was highest for moss, intermediate for soil and lowest for litter. RM_{10} was higher at 25 and 35 °C than at 15 °C, or was higher in DM2 than in DM1 (Fig. 1c).

The main effect of temperature was significant on the rates of litter (Fig. 2b) and soil BNF (Fig. 2c), but not on moss BNF rate (Fig. 2a). The main effects of moisture were significant on BNF rates in all the three substrates (Fig. 2). The interactive effect of temperature × moisture was marginally significant ($P < 0.1$) on moss BNF rate, but was significant on litter or soil BNF rate. The temperature responses of moss BNF rate were moisture dependent, with the rate highest at 15 °C and lowest at 25 °C under LM, but higher at 35 °C than at 15 °C under HM (Fig. 2a). Both litter and soil BNF rates increased with temperature under the three moisture levels (Fig. 2b and c). At each temperature, the BNF rates increased with moisture for moss and litter (Fig. 2a and b). However, the patterns of the moisture responses were different among the three temperatures for soil BNF rate (Fig. 2c). The rate was not changed by moisture at 15 °C, increased significantly with moisture at 25 °C, while the rate was greatest under MM, but lowest under LM at 35 °C (Fig. 2c).

Based on two-way ANOVA, the temperature sensitivity (Q_{10}) of BNF for the three substrates was significantly ($P < 0.05$) or marginally ($P < 0.1$) affected by ΔT and moisture separately or in combination except for the Q_{10} for litter BNF ($P > 0.1$) (Fig. 3). For moss, a significant effect of ΔT was found under LM with the Q_{10} value in DT2 higher than that in DT1 (Fig. 3a). For litter, a significant difference in Q_{10} between the two temperature ranges was only found under HM with the value higher in DT2 than in DT1 (Fig. 3b). For soil, significant effects of ΔT were found under MM and HM, but the patterns were

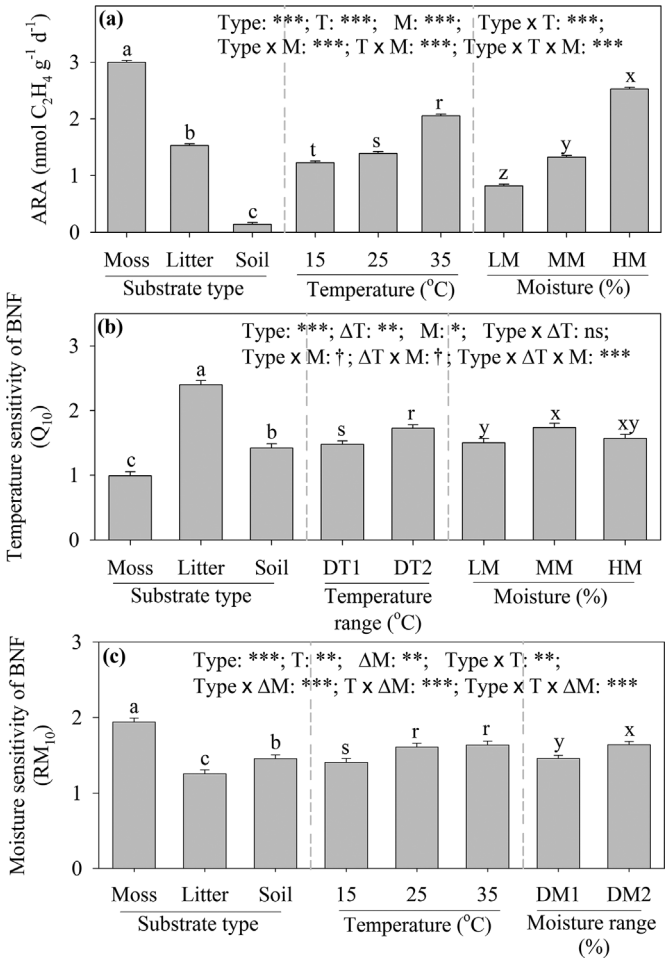
(a)
Type: ***; T: ***; M: ***; Type x T: ***;
Type x M: ***; T x M: ***; Type x T x M: ***
ARA (nmol C_2H_4 g^{-1} d^{-1})
Moss Litter Soil
Substrate type
15 25 35
Temperature (°C)
LM MM HM
Moisture (%)
(b)
Type: ***; ΔT: **; M: *; Type x ΔT: ns;
Type x M: †; ΔT x M: †; Type x ΔT x M: ***
Temperature sensitivity of BNF (Q_{10})
Moss Litter Soil
Substrate type
DT1 DT2
Temperature range (°C)
LM MM HM
Moisture (%)
(c)
Type: ***; T: **; ΔM: **; Type x T: **;
Type x ΔM: ***; T x ΔM: ***; Type x T x ΔM: ***
Moisture sensitivity of BNF (RM_{10})
Moss Litter Soil
Substrate type
15 25 35
Temperature (°C)
DM1 DM2
Moisture range (%)

Fig. 1. Main effects of substrate type, temperature/or temperature range (ΔT) and moisture/or moisture range (ΔM) on (a) free-living biological N_2 fixation rate (nmol C_2H_4 g^{-1} mass d^{-1}), and (b) temperature sensitivity (Q_{10}) of biological N_2 fixation, and (c) moisture sensitivity (RM_{10}) of biological N_2 fixation. Bars represent mean ± standard error. DT1 and DT2 represent the temperature ranges of 15–25 °C and 25–35 °C, respectively; and DM1 and DM2 denote the moisture ranges of LM-MM and MM-HM, respectively. LM, MM and HM denote low, mid and high levels of moisture, respectively. Different letters denote significant difference at $P < 0.05$ among substrate types, temperature or moisture levels or between temperature (or moisture) ranges (three-way ANOVA with Tukey's *post-hoc* test). ns: not significant; †: marginally significant at $P < 0.1$ level; *, ** and *** denote significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels, respectively.

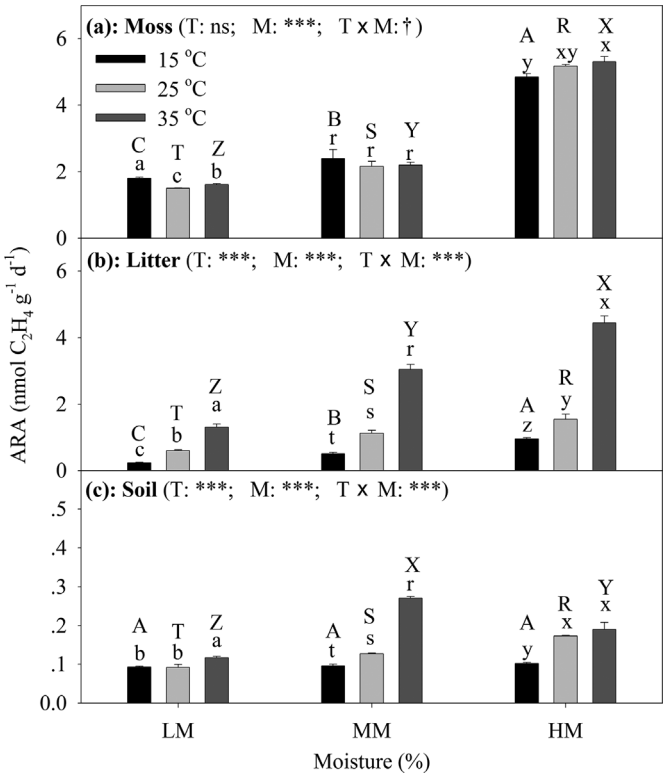

Fig. 2. The responses of free-living biological N_2 fixation to temperature (or moisture) under each moisture level (or at each temperature) for (a) moss, (b) litter and (c) soil. Bars represent mean ± standard error. LM, MM and HM denote low, mid and high levels of moisture, respectively. Different lowercase letters represent significant differences at $P < 0.05$ among the three temperatures under each moisture level (one-way ANOVA with Tukey's *post-hoc* test). Different capital letters denote significant differences at $P < 0.05$ among the three moisture levels at each temperature (one-way ANOVA with Tukey's *post-hoc* test). The data in the parentheses behind each panel label represent the results from two-way ANOVA showing the effects of temperature (T), moisture (M) and their possible interaction (T × M) on BNF rate (ns: not significant; †: marginally significant at $P < 0.1$ level; *, ** and *** denote significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels, respectively).

reversed with the values greater in DT2 and DT1 under MM and HM, respectively (Fig. 3c). The moisture effects on Q_{10} were different between the two temperature ranges for each substrate (Fig. 3). In DT1, the Q_{10} values increased with moisture for moss and soil BNF (Fig. 3a, c), but the pattern was reversed for litter BNF (Fig. 3b). In DT2, a significant moisture effect was only found for soil BNF with the Q_{10} value higher under MM than under LM and HM (Fig. 3c).

The main effect of temperature or temperature and ΔM in combination on RM_{10} was only significant for soil BNF (Fig. 4). The main effects of ΔM on RM_{10} were significant for moss and soil BNF (Fig. 4a, c). For moss BNF, RM_{10} was greater under DM2 than DM1 at the three temperatures (Fig. 4a). A significant moisture effect was also found at 35 °C for soil BNF with the value greater in DM1 than DM2 (Fig. 4c). Temperature only had significant effects on RM_{10} for soil BNF, with the value greater at 35 °C than at 15 and 25 °C in DM1, while highest at 25 °C and lowest at 35 °C in DM2 (Fig. 4c).

4. Discussion

4.1. Temperature responses of nitrogen fixation in soil, litter and moss

The present study shows that the BNF rate increases with temperature for both soil and litter (Fig. 2b and c). This is in line with previous studies (Gentili et al., 2005; Houlton et al., 2008; Gundale et al., 2012a; Lindo et al., 2013; Diáková et al., 2016; Rousk et al., 2017), which show that BNF rate changes with temperature in a quadratic way, where the rate increases with temperature until reaching a maximum value with the optimum temperatures varying from 20 to 30 °C. In the current study, soil and litter BNF rates increase with temperature within the included temperature range from 15 to 35 °C. This is in line with the aforementioned studies, but the optimum temperatures exceed 30 °C. Similarly, studies referenced by Houlton et al. (2008) exhibit optimum temperatures around 35 °C. One possible reason for the relatively high optimum temperatures in the current study is that the samples were collected from a subtropical region, where air temperature often exceeds 35 °C in the summer. Hence, the free-living diazotrophs have likely adapted to the local climate. Different species of diazotrophs may play an important role in determining optimal BNF temperature. For example, different species of cyanobacteria were used to explain the differential temperature responses of moss BNF (Gentili et al., 2005; Gundale et al., 2012a; Rousk et al., 2017). Thus, the relatively high optimum temperature observed in the current study may be due to different species of diazotrophs between

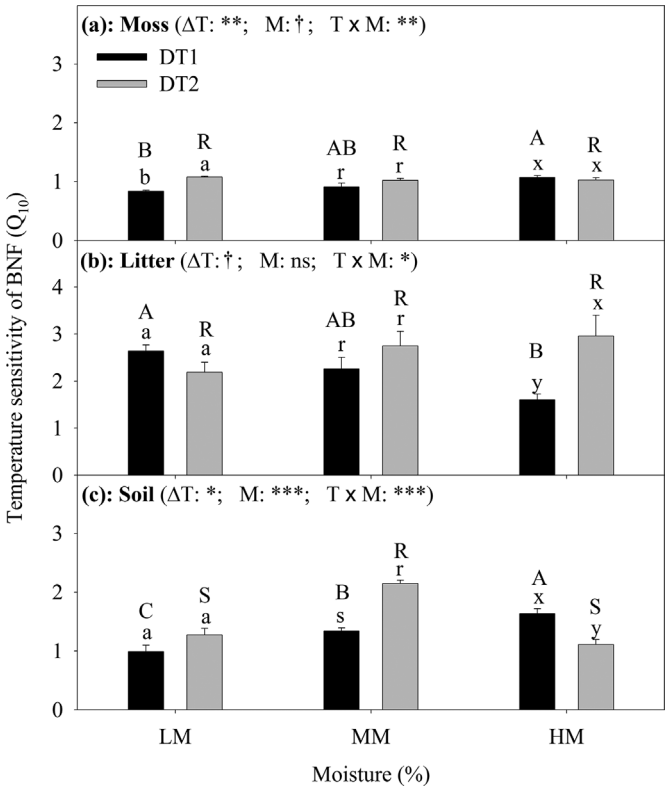

Fig. 3. The responses of temperature sensitivity (Q_{10}) of BNF to temperature range (ΔT) or moisture under each moisture level (or in each temperature range) for (a) moss, (b) litter and (c) soil. Bars represent mean ± standard error. LM, MM and HM denote low, mid and high levels of moisture, respectively. Different lowercase letters represent significant difference at $P < 0.05$ between the two temperature ranges under each moisture level (independent-samples T test). Different capital letters denote significant difference at $P < 0.05$ among the three moisture levels in each temperature range (one-way ANOVA with Tukey's *post-hoc* test). The data in the parentheses behind each panel label represent the results from two-way ANOVA showing the effects of temperature range (ΔT), moisture (M) and their possible interaction (ΔT × M) on Q_{10} (ns: not significant; †: marginally significant at $P < 0.1$ level; *, ** and *** denote significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels, respectively).

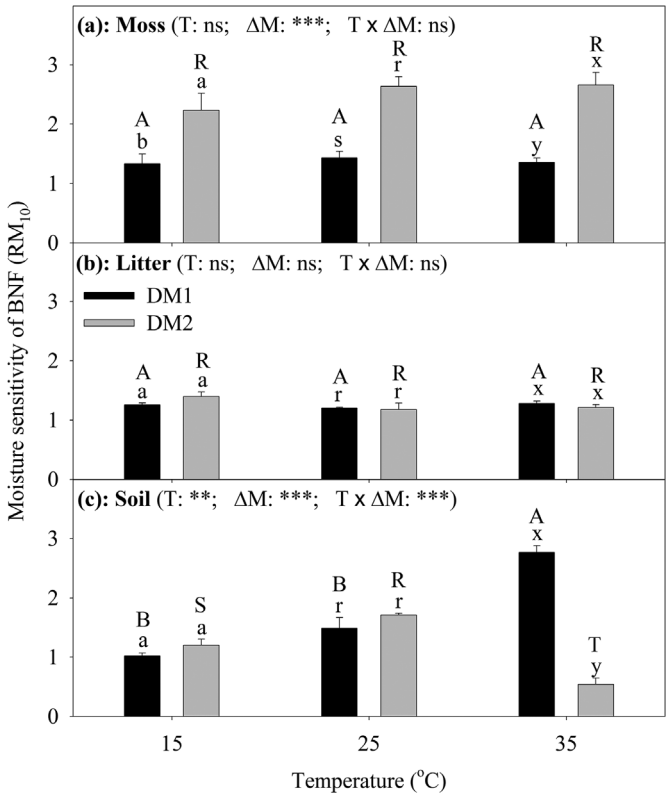
(a): Moss (T: ns; ΔM: ***; T x ΔM: ns)
(b): Litter (T: ns; ΔM: ns; T x ΔM: ns)
DM1
DM2
(c): Soil (T: **; ΔM: ***; T x ΔM: ***)
Moisture sensitivity of BNF (RM10)
15
25
35
Temperature (°C)

Fig. 4. The responses of moisture sensitivity (RM_{10}) of BNF to moisture range (ΔM) or temperature at each temperature (or in each moisture range) for (a) moss, (b) litter and (c) soil. Bars represent mean ± standard error. DM1 and DM2 denote moisture ranges from LM to MM, and from MM to HM, respectively. Different lowercase letters represent significant difference at $P < 0.05$ between the two moisture ranges at each temperature (independent-samples T test). Different capital letters denote significant difference at $P < 0.05$ among the three temperatures in each moisture range (one-way ANOVA with Tukey's *post-hoc* test). The data in the parentheses behind each panel label represent the results from two-way ANOVA showing the effects of temperature (T), moisture range (ΔM) and their possible interaction (T × ΔM) on RM_{10} (ns: not significant; *, ** and *** denote significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels, respectively).

the subtropical regions and other regions with higher latitudes. The consistent increase with temperature for BNF rates in soil and litter (Fig. 2b and c) suggests that N inputs will be enhanced with warming across a broad range of precipitation regimes.

However, we find that the effect of temperature on moss BNF rate is dependent on moisture (Fig. 2a). This is likely due to the impact of desiccation, which may occur when temperature increases under low and intermediate moisture for moss (Gundale et al., 2012a, 2012b; Lindo et al., 2013). The negative effect of desiccation may exceed the positive effect of temperature on moss BNF rate under low and intermediate moisture conditions (Gundale et al., 2012b). This can well explain the decrease or negligible change of moss BNF rate under low and intermediate moisture conditions (Fig. 2a). However, the desiccation stress can be partly overcome under high moisture conditions, and in turn moss BNF rate increases with temperature under high moisture as observed in the current study (Fig. 2a). Similarly, an increase of moss BNF rate with temperature has been reported when moisture is not limiting (Gundale et al., 2012a). Previous studies also reported no change or occasionally decrease in moss BNF rate under warming (Deslippe et al., 2005; Sorensen and Michelsen, 2011; Sorensen et al., 2012; Whiteley and Gonzalez, 2016). According to our study, moisture may play a role in determining the different temperature response patterns for moss BNF rate in these studies. As the low level of moisture (LM) for moss was designed to be the annual average in the studied forest, our findings suggest that N input via moss BNF in the studied forest is likely to decrease under global warming if the current precipitation regime persists. Considering that moss BNF rate only increases with temperature under high moisture conditions (Fig. 2a: HM), and different areas are expected to be drier or wetter in the southwest China (Liu et al., 2014), N input via moss BNF may only be enhanced in areas with increased precipitation under global warming.

The temperature sensitivity (Q_{10}) is an important index reflecting the temperature response of an enzyme-dominated process (Wallenstein et al., 2011). The temperature sensitivities of BNF for different substrates have rarely been measured, although they are crucial for predicting N input via BNF under global warming. According to the present study, the Q_{10} values of BNF differed with substrate being greatest for litter, intermediate for soil, and lowest for moss, suggesting different Q_{10} values should be integrated into terrestrial ecosystem models, in which the current modeling of BNF is entirely empirical (William et al., 2015). As for the studied forest, although the averaged BNF rate for moss was 2.0 and 21.4 times those in litter and soil, respectively, significant differences in the area-based BNF rate (6.8 ± 0.4 μg N m^{-2} d^{-1} in soil, 14.3 ± 1.2 μg N m^{-2} d^{-1} in litter, and 11.3 ± 1.8 μg N m^{-2} d^{-1} in moss) was only found between soil and litter, higher in the latter (Fig. S2). Given the Q_{10} values were much higher for litter BNF (2.4 ± 0.07) and soil (1.4 ± 0.07) than for moss BNF (1.0 ± 0.07) across the data, N input via BNF in the studied forest would increase substantially under global warming. It should be noted that, although within the reported range, the measured BNF rates were relatively low compared to the reported values in the tropical/subtropical forests

(Note S1).

Compared to the available data, the Q_{10} values for moss BNF observed in the current study were within the range (from 0.33 to 7.51) for BNF in two mosses (*Pleurozium schreberi* and *Tomentypnum nitens*) from temperate heath, subarctic tundra and arctic tundra (Rousk et al., 2017). According to their study, the Q_{10} values of moss BNF varied greatly, and were affected by both temperature and moisture (Rousk et al., 2017). Similarly, our results show that the Q_{10} values for the three substrates are modulated by moisture and/or temperature range (Fig. 3), suggesting that the effects of temperature and moisture should be considered when integrating Q_{10} into terrestrial ecosystem models.

4.2. Moisture responses of nitrogen fixation in soil, litter and moss

Compared to temperature, limited studies have been conducted to investigate the precipitation/moisture effects on BNF either separately or in combination with temperature (Gundale et al., 2009; Whiteley and Gonzalez, 2016; Rousk et al., 2017). It has been reported that the optimal moisture conditions for BNF are near saturation and BNF rate decreases linearly as moisture lowers (Vitousek et al., 2002). This is supported by our results, where a significant increase in moss and litter BNF rates occurred with increasing moisture regardless of temperature (Fig. 2a and b). This implies that the effects of precipitation on moss and litter BNF rates (e.g., enhanced by increasing precipitation or suppressed by decreasing precipitation) are likely widespread in different seasons or areas with different air temperatures or under global warming. Nevertheless, for soil BNF rate, the different patterns (i.e., no change with moisture at 15 °C, increase with moisture at 25 °C, while increase initially followed by a decrease at 35 °C (Fig. 2c)) at low and high temperatures may be related to diazotrophs' activity and substrate availability, respectively. The low temperature treatment (15 °C) is lower than the mean annual temperature, especially the temperature in July, when the samples were collected. The diazotrophs' activity may have been suppressed by low temperature, and thus is probably insensitive to moisture change (Fig. 2c). Under the high temperature treatment, the diazotrophs' activity should theoretically increase with soil moisture. Nevertheless, a simultaneous increase in soil moisture may have limited diffusion of N_2 (the water-filled pore space (WFPS) was about 80% with a bulk density of 1.1 g cm^{-3}) into the sites with diazotrophs, which subsequently lead to an apparent decrease of soil BNF rate. In support of this, soil BNF rates were similar under high moisture conditions at 25 and 35 °C (Fig. 2c).

While moisture responses of BNF have been received limited attention (Jackson et al., 2011; Gundale et al., 2012b; Rousk et al., 2017), an index quantifying moisture sensitivity has never been proposed. In the current study, moisture sensitivity (RM_{10}) was quantified in a similar way as temperature sensitivity (Q_{10}). A similar idea has been used to quantify moisture sensitivity of soil respiration (Jiang et al., 2013). Our results show that RM_{10} exhibits a contrasting pattern to Q_{10} for BNF in the three substrates, with the RM_{10} value highest for moss BNF but lowest for litter BNF (Fig. 1c). Therefore, N inputs via moss BNF would increase or decrease most pronouncedly as precipitation increase (or decrease).

Additionally, our results show that differences exist in terms of the moisture responses between moss and litter BNF (Fig. 4a and b), though both the moss and litter BNF rates increased with moisture at the three temperatures (Fig. 2a and b). Moss BNF is more sensitive to moisture change under wetter than drier conditions (Fig. 4a), but litter BNF is similarly sensitive to moisture changes regardless of dry or wet conditions according to their patterns of RM_{10} (Fig. 4b). This suggests that moss BNF would increase disproportionately when the climate becomes wetter, or *vice versa*. However, the fact that the moisture responses for both moss and litter BNF are not modulated by temperature (Fig. 4a and b) suggests that the current moisture responses will persist under global warming. For soil, BNF is less sensitive (i.e., lower RM_{10}) to moisture changes under the wetter conditions (Fig. 4c), probably due to limited diffusion of N_2 into the sites with diazotrophs. Different from moss and litter BNF rates, our results suggest that soil BNF rate would be more sensitive to moisture changes in warmer conditions given that soil moisture does not limit diffusion of N_2 (Fig. 4c). Therefore, a moderate soil moisture would likely benefit soil BNF under global warming.

In summary, the responses of BNF rates in moss, litter and soil to temperature and moisture were divergent. The litter and soil BNF rates increased with temperature irrespective of moisture levels, but the moss BNF rate was only increased under high moisture. The moss and litter BNF rates increased with moisture regardless of temperature levels, but the responses of soil BNF rate to moisture were modulated by temperature. In general, the temperature sensitivity (Q_{10}) was highest for litter, intermediate for soil and lowest for moss, but the moisture sensitivity (RM_{10}) was highest for moss, intermediate for soil and lowest for litter. Our findings suggest that different temperature or moisture sensitivities for the three substrates should be integrated into terrestrial ecosystem models in order to better predict N inputs via BNF under climate change. However, it should be noted that some uncertainties are involved as the findings are derived from a single, short-term laboratory based experiment. Firstly, we are not sure whether the findings are applicable to the whole karst region of southwest China, or to other regions in the subtropical and tropical climate zones. Secondly, there is uncertainty in terms of whether the patterns found in the current study are representative for BNF in moss, litter and soil in general, since only one species of moss, one litter type (leaf litter) and one soil type (calcareous lithosol) are included. Thirdly, the question remains: are the responses of BNF to temperature and moisture observed in the short-term experiment comparable to responses under long-term climate change? Because of the uncertainties, more investigation is urgently needed in order to better predict BNF responses to climate change.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.11.026.

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